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Apolipoprotein E expression at neuromuscular junctions in mouse, rat and human skeletal muscle

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Abstract Apolipoprotein E (ApoE)-ε4 allele has been associated with late onset familial Alzheimer's disease (AD). In both familial and sporadic AD brain, ApoE is localized to the vessel walls, senile amyloid plaques, and neurofibrillary tangles. ApoE is also an 'injury-response' macromolecule in peripheral nerves and was reported to increase in response to injury. We have demonstrated that Alzheimer β-amyloid precursor protein and a serpin α_1-antichymotrypsin also found accumulated in senile plaques in AD brain, were also localized at neuromuscular junctions (NMJs). Using immunocytochemistry, our present results indicate that ApoE is found in normal mouse, rat and human skeletal muscle and concentrated at the NMJs as it is in the senile plaques in AD brain. Such experiments may shed light on the mechanism of synapse loss, as well as plaque formation in this neurodegenerative disease.

Key words: Cholinergic synapse; Skeletal muscle; Apolipoprotein E; Serine protease inhibitor; Alzheimer's disease

1. Introduction

Apolipoprotein E (ApoE) is a 299 amino acid plasma protein which has been found in Alzheimer senile plaques, vascular amyloid and neurofibrillary tangles [1]. It is encoded by 4 exons of a gene located on human chromosome 19 [2]. There are 3 major isoforms, ApoE2, E3 and E4, each differing from the other by only 2 amino-acids [3], encoded by 3 different alleles ε2, ε3 and ε4 [4]. The ApoE-ε4 allele is reported to correlate best with Alzheimer pathology in late-onset familial [5,6] and may convey increased risk in the sporadic disease [7]. Dystrophic neurites are an important and a consistent pathological finding associated with senile plaques in AD [1]. Synapse loss, previously predicted to be the site of dysfunction in AD [8] has more recently been correlated with the severity of the symptoms and the importance of the dementia as quantified by neuropsychological testing [9,10].

In an effort to understand the mechanisms involved in this synaptic alteration, we have utilized the neuromuscular junction (NMJ) as a convenient model for cholinergic nicotinic synapse studies (see [11]). Originally proposed for synaptic degeneration in the pathogenesis of amyotrophic lateral sclerosis [12], more recent studies have shown that β-amyloid protein precursor, α_1-antichymotrypsin and protease nexin I, three serine protease inhibitors that accumulate in the brain of AD patients, were localized at normal NMJs [13,14]. In addition, the synapse-organizing macromolecule, agrin, contains serine protease inhibitor domains (Kazal-type) and is a protease inhibitor [15].

Since ApoE has been associated with β-amyloid peptide in AD brain [5], we decided to investigate whether ApoE was also present at NMJs junctions of skeletal muscle using immunocytochemical studies with a polyclonal antibody raised against ApoE. We now demonstrate that ApoE is expressed in all NMJs of mouse, rat and human skeletal muscle.

2. Materials and methods

Biopsy material from the hippocampus of a familial AD patient was used after fixation of the whole brain in 4% neutral-buffered formalin for 4 weeks. Tissue blocks were embedded in paraffin.

Deltoid muscle biopsies were obtained from adult subjects without detectable skeletal muscle disease and were frozen without fixation by quick immersion in isopentane chilled in liquid nitrogen and stored at −80°C until used. Adult BALB/c mice and Wistar rats (Centre d'Élevage R. Janvier, Le Genest, France), were killed by cervical dislocation, and the gastrocnemius muscles removed. These specimens were frozen as above.

2.1. *ApoE immunocytochemistry and acetylcholine receptor visualization*

For immunocytochemistry, 8 μm transverse cryostat or paraffin sections were made and deposited on gelatin-subbed microscope slides. A polyclonal antibody made against purified ApoE (Cortex Biochem Inc., San Leandro, CA) diluted 1:100 in phosphate buffered saline (PBS) containing 0.3% Triton X-100 (PBST) and 1% bovine serum albumin (BSA) was incubated for 2 h at room temperature.

Sections were further incubated for one hour in anti-goat biotinylated F $(ab')^2$ fragments of IgG (Amersham, Les Ulis, France) diluted 1:100 in PBST. They were incubated in fluorescein isothyocyanate (FITC)-tagged streptavidin (Amersham) diluted 1:100 in PBST for 1 h and lastly with tetramethyl rhodamine-labeled α-bungarotoxin (Molecular Probes) diluted 1:1,000 to identify acetycholine receptors (AChRs) on NMJs as previously described [14]. Omission of primary antibody or replacing primary antibody with non-immune goat serum were used as negative controls. Brain tissue from a case of familial AD was taken as a positive control and processed for ApoE immunocytochemistry as above.

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Abbreviations: ApoE, apolipoprotein E; Alzheimer's disease, AD; NMJ, neuromuscular junction.

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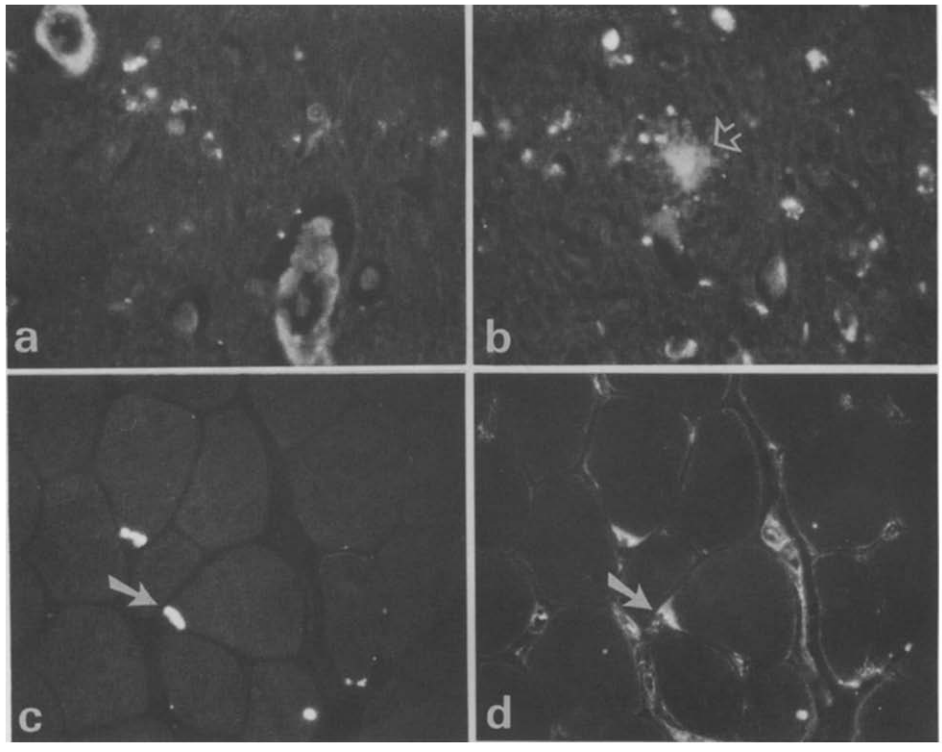

Fig. 1. (a,b) Immunolocalization of ApoE in AD brain paraffin sections. Note the staining by anti-ApoE antibody of vessel walls (a) and senile plaques (b; empty arrow). Magnification × 230. (c,d) Immunolocalization of ApoE (d) in human skeletal muscle cryostat sections at NMJ (arrow) an in the endomysial vessel walls. NMJs are identified by tetramethyl-rhodamine α-bungarotoxin (c) on the same section. Magnification × 168.

3. Results

3.1. Immunocytochemical detection of ApoE in the brain of AD patients

ApoE immunolabelling was observed in the vessel walls and senile plaques in AD brain tissue (Fig. 1a,b).

3.2. Immunocytochemical detection of ApoE in normal adult mouse, rat and human skeletal muscle

In normal human skeletal muscle, immunocytochemical staining with antibody against ApoE showed prominent labeling of all NMJs identified by tetramethylrhodamine-labeled α-bungarotoxin (Fig. 1c,d). In addition, ApoE was also detected in endomysial vessel walls.

In normal mouse (Fig. 2a,b) and rat skeletal muscle (Fig. 2c,d) a similar immunolabeling was observed.

4. Discussion

In the present study, we present evidence, for the first time to our knowledge, that ApoE is present in mouse, rat and human skeletal muscle where it is found in endomysial vessel walls and highly concentrated at the NMJ. ApoE at the NMJ may originate from the motoneuron and be transported within axons. ApoE expression in normal nerve has been interpreted to indicate its important role in myelinogenesis where it might mobilize lipids into or within the myelin [16]. It may also be, that, like protease nexin I [13], ApoE is synthesized in muscle, Schwann cells and/or, possibly, macrophages [17] and endothelial cells. Apo E has previously been detected in non-myelinating Schwann cells [18]. It is equally possible that NMJ-associated ApoE arises from Schwann cells, from the nerve or the muscle cell. Precisely where at the synapse, pre- or post-synaptic membrane or basement membrane, ApoE is located will require ultrastructural analysis.

The presence of ApoE at NMJs might arise through its interaction with the α_2-macroglobulin receptor/low density lipoprotein receptor-like protein (α2macR/lrp) which serves to bind α_2-macroglobulin, a potent protease inhibitor [19], and elastase (a serine protease) complex. Other serine protease:serpin complexes, such as urokinase and plasminogen activator inhibitor-1 (PAI-1) complexes, also bind to the α2macR/lrp [20]. In this respect, we have shown that other protease inhibitors such as protease nexin I, protease nexin II, and α_1-antichymotrypsin, are present at the NMJ [13,14]. These serine protease inhibitors are also concentrated at senile amyloid plaques in the brain of AD patients, like ApoE. Protease nexin II is identical to the secreted forms of the β-amyloid protein precursor, a potent inhibitor of chymotrypsin and the coagulation factors XIa and IXa [21], and the principal component of senile plaques. ApoE is also know to bind to β-amyloid peptide, although its precise interaction is unclear [5]. ApoE, which is also a heparin binding protein [22], could promote cell-matrix interactions and stimulate axon extension. It has also a role in membrane lipid biosynthesis of all cells, not only nerve, and there is evidence that Schwann cells, like cortical astrocytes [18,23] or the peripheral nerve macrophages during Wallerian degeneration [17,24] produce increased amounts of ApoE.

Our present findings support previous studies which suggest that inhibitors of serine proteases are involved in synaptic stabilization [12,25,26]. The synapse has been considered an important site underlying mechanisms of degeneration in AD for

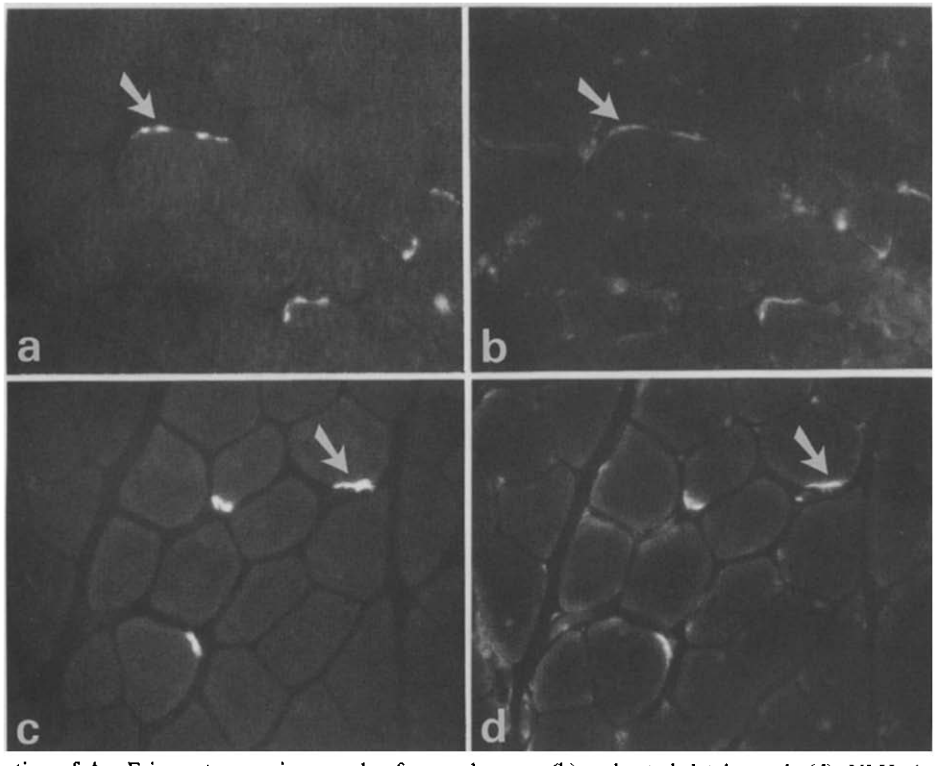

Fig. 2. Immunolocalization of ApoE in gastrocnemius muscle of normal mouse (b) and rat skeletal muscle (d). NMJs (arrow) are identified by tetramethyl-rhodamine α-bungarotoxin (a and c). Magnification × 168.

more than 20 years (see [8]). Recent evidence has supported and extended this supposition [9,10] and shown that synapse loss correlates better with dementia than does deposition of either amyloid plaques or presence of neurofibrillary tangles. Since ApoE is present within senile plaques in AD brains and at normal peripheral, cholinergic synapses, studies in muscle may further help our understanding of the mechanism of synapse loss, as well as plaque deposition, in this disease.

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